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Acetylcholine modulates gamma frequency oscillations in the hippocampus by activation of muscarinic M1 receptors

Running title: M1 receptors regulate gamma oscillations

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Abstract

Modulation of gamma oscillations is important for the processing of information and the disruption of gamma oscillations is a prominent feature of schizophrenia and Alzheimer's disease. Gamma oscillations are generated by the interaction of excitatory and inhibitory

neurons where their precise frequency and amplitude are controlled by the balance of
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excitation and inhibition. Acetylcholine enhances the intrinsic excitability of pyramidal neurons and suppresses both excitatory and inhibitory synaptic transmission but the net modulatory effect on gamma oscillations is not known. Here, we find that the power, but not frequency, of optogenetically-induced gamma oscillations in the CA3 region of mouse hippocampal slices is enhanced by low concentrations of the broad spectrum cholinergic agonist carbachol but reduced at higher concentrations. This bidirectional modulation of gamma oscillations is replicated within a mathematical model by neuronal depolarization, but not by reducing synaptic conductances, mimicking the effects of muscarinic M1 receptor activation. The predicted role for M1 receptors was supported experimentally; bidirectional modulation of gamma oscillations by acetylcholine was replicated by a selective M1 receptor agonist and prevented by genetic deletion of M1 receptors. These results reveal that acetylcholine release in CA3 of the hippocampus modulates gamma oscillation power but not frequency in a bidirectional and dose-dependent manner by acting primarily through muscarinic M1 receptors.

Introduction

Gamma oscillations are synchronous network oscillations in the 30-100Hz range found throughout the neocortex and hippocampus. The entrainment of neuronal activity to this high frequency oscillation is thought to be important for the timing of spikes both within and between different brain structures determining the flow of information (Colgin et al., 2009; Sohal et al., 2009; Ainsworth et al., 2012). The occurrence of correlated or coherent activity at specific behavioral time points is a critical feature of attention and sensory processing (Fries et al., 2001; Womelsdorf et al., 2006) and underlies synaptic plasticity required for the encoding of long-term memory (Kwag and Paulsen, 2009). Moreover, perturbations of

gamma oscillation frequency, power and coherence are found in several cognitive disorders including schizophrenia and Alzheimer's disease (Cho et al., 2006; Verret et al., 2012; Gonzalez-Burgos et al., 2015) indicating the importance of gamma oscillations for cognitive functions .

Generation of gamma oscillations *in vivo* can be achieved by reciprocally connected populations of excitatory and inhibitory neurons (Penttonen et al., 1998; Atallah and Scanziani, 2009; Cardin et al., 2009; Sohal et al., 2009) (see Figure 4A). This anatomical arrangement occurs throughout the neocortex and hippocampus where inhibitory fast-spiking parvalbumin positive basket cells (PV BCs) provide feedback inhibition of excitatory pyramidal cells (PCs). In the hippocampus gamma oscillations are commonly observed “nested” on the phase of theta oscillations (Lisman and Jensen, 2013) but *in vitro* gamma oscillations may also be induced by application of glutamatergic or cholinergic agonists (Fisahn et al., 1998; Palhalmi et al., 2004). Gamma oscillation phase can be entrained by strong excitatory or inhibitory input to the network (Akam et al., 2012) which is proposed to be a mechanism for the generation of different frequencies of gamma within the same brain region (Colgin et al., 2009; Jadi and Sejnowski, 2014; Lasztoczi and Klausberger, 2014; Schomburg et al., 2014). However, in comparison to their generation, much less is known about the mechanisms for modulation of gamma oscillations.

The release of acetylcholine in the neocortex and hippocampus activates muscarinic and nicotinic receptors that regulate the processing of information within these circuits (Hasselmo, 2006; Teles-Grilo Ruivo and Mellor, 2013). Muscarinic and nicotinic receptors are targeted to specific compartments that enable each subtype to control the function of selective nodes within a circuit. For example, M1 muscarinic receptors are principally located on somatic and dendritic compartments of PCs (Levey et al., 1995; Yamasaki et al., 2010), with some evidence for expression in interneurons (Cea-del Rio et al., 2010; Yi et al., 2014),

where they increase excitability by causing the opening of non-selective cationic channels and inhibiting K^+ channels **such as M channels and SK channels** (Madison et al., 1987; Fisahn et al., 2002; Buchanan et al., 2010). Genetic deletion of M1 receptors or pharmacological inhibition of muscarinic receptors disrupts memory (Blokland et al., 1992; Anagnostaras et al., 2003; Atri et al., 2004; Wess, 2004; Green et al., 2005) whereas administration of muscarinic receptor agonists or acetylcholinesterase inhibitors in Alzheimer's disease (Bodick et al., 1997; McGleenon et al., 1999) or muscarinic receptor agonists in cognitively impaired humans (Shekhar et al., 2008; Nathan et al., 2013) can improve memory. Cholinergic agonists and acetylcholine esterase inhibitors induce gamma oscillations *in vitro* (Fisahn et al., 2002; Spencer et al., 2010) but their role in modulating pre-existing gamma oscillations and the cholinergic receptor subtypes involved are less well characterised.

To study the mechanisms underlying the modulation of gamma oscillations we made use of *in vitro* and *in silico* models of gamma oscillations. These systems have previously been used to investigate the mechanisms for the generation of gamma oscillations but have rarely been employed to determine the mechanisms by which they might be modulated. Using optogenetically-induced gamma oscillations in hippocampal CA3 and a mathematical network model we show that acetylcholine regulates the power, but not frequency, of gamma oscillations in a bidirectional, dose-dependent manner which is mediated by activation of muscarinic M1 receptors.

Materials and Methods

Ethical approval

All experiments were performed in accordance with the UK Animal Scientific Procedures Act (1986) and local guidance from the Home Office Licensing Team at the University of Bristol. The protocol was approved by the Animal Welfare and Ethics Review Board at the University of Bristol.

Transfection

53 Wild-type, male, C57/BL6 mice or muscarinic M1 receptor KO mice (M1 KO, bred on a C57/BL6 background; line 1784, Taconic (Fisahn et al., 2002)) were used with experiments interleaved for wild-type and M1 KOs. p21 – 24 wild-type mice or p25 – 40 M1 KO mice underwent stereotaxic surgery to inject virus into the CA3 region of the hippocampus. Under inhalation anaesthesia (1-3% O₂, 0.5-2% isoflurane) animals were given stereotaxic injections of rAAV5-CaMKIIa-hChR2 (H134R)-EYFP virus (Virus Vector Core; 0.5 µl of 4×10^{12} T.U. ml⁻¹) at the following coordinates: (from bregma, in mm) posterior 2.3, lateral 2.2, ventral 2.2 (wild-type mice) and posterior 2.46 to 2.80, lateral 2.60 to 2.85, ventral 2.36 to 3.00 (M1KO mice, coordinates chosen based on animal age). To assess construct expression, animals were anaesthetized with intraperitoneal injection of sodium pentobarbital (150 mg/kg) and perfused transcardially with 4% paraformaldehyde (Sigma-Aldrich) at 7, 21 and 35 days after transfection. 60 µm thick brain sections were taken with the aid of a freezing microtome and mounted with Vectashield medium (Vector laboratories) containing DAPI allowing the visualisation of cell nuclei alongside ChR2-YFP expression under an epifluorescence microscope.

Slice electrophysiology

Acute hippocampal slices were prepared 7 - 40 days after transfection for initial characterization and 27 – 53 days after transfection for all drug concentration comparisons. Animals were decapitated following cervical dislocation, the brain removed and hippocampi dissected in ice-cold modified ACSF containing (in mM): 252 sucrose, 2.5 KCl, 26 NaHCO₃, 1 CaCl₂, 5 MgCl₂, 1.25 NaH₂PO₄ and 10 glucose. Transverse hippocampal slices of 400 µm thickness were cut using a microslicer (Leica VT1200S) and stored in standard ACSF containing (in mM): 119 NaCl, 10 glucose, 26 NaHCO₃, 2.5 KCl, 1 NaH₂PO₄, 2.5 CaCl₂ and 1.3 MgSO₄ at room temperature for at least 1 h prior to recording. All solutions were saturated with 95% O₂, 5% CO₂.

For recording, slices were transferred to a dual perfusion style submerged chamber for increased metabolic supply (RC-27L, Warner Instruments). Standard ACSF was perfused at a rate of 8.5 ml min⁻¹ and recording chamber temperature was maintained at 32-34°C. Slices were visualised using infrared differential interference-contrast or fluorescence microscopy and YFP fluorescence excited with a 505 nm wavelength LED. Channelrhodopsin was excited with a 470 nm wavelength LED through the 4x objective on the microscope. Local field potential (LFP) recordings were made using borosilicate glass pipettes with resistance 3-6 MΩ when filled with standard ACSF. LFPs were recorded with a multiclamp 700A amplifier (Molecular devices), filtered with a Bessel low-pass filter at 200 Hz and sampled at 10 kHz using a Micro 1401 data acquisition board (CED). No correction was made for background 50 Hz noise. Recordings were made using Signal2 software (CED) and analysed offline using custom written programs in MATLAB.

Mathematical model

The model for a single cell (node in the network) was based on the model of Kopell et al. (Kopell et al., 2010) which uses the Hodgkin-Huxley equation (1)

$$C \frac{dV}{dt} = g_{Na} m_{\infty}(V)^3 h(V_{Na} - V) + g_K n^4 (V_K - V) + g_L (V_L - V) + I_{Km} + I_{syn} + I \quad (1)$$

$$I_{Km} = g_{Km} k_{Km} (V_{Km} - V) \quad (2)$$

The membrane potential of both pyramidal cells and interneurons is governed by the interaction of sodium (Na), potassium (K) and leak currents (L) as well as an applied current (I). Pyramidal cells also contain an additional m-current potassium conductance modelled as in (Nowacki et al., 2011) and using $V_{Km} = -35\text{mV}$: equation (2). The applied current takes a variety of forms including step, ramp and sine wave functions. In the model, capacitance density (C) is measured in $\mu\text{F cm}^{-2}$, voltage (V) in mV and time (t) in ms. The variables g_{Na} , g_K and g_L are the maximal ionic conductances (mS cm^{-2}). m , h and n are rate functions which determine the gating characteristics of the respective channel and are identical to those given in (Kopell et al., 2010). The gating variables are different between pyramidal and interneurons and determine their distinct firing properties. Initial model parameters based on (Kopell et al., 2010) are given in Table 1.

Cells were synaptically coupled in an all-to-all configuration using the following expression:

$$I_{syn} = g_{ij} s_i(t) (V_{rev} - V_j) \quad (3)$$

Where g_{ij} is the maximal conductance of the synapse, s_i is the gating variable and V_{rev} is the reversal potential of the synapse. Finally, there was stochastic external drive applied to the network to simulate noise. A network of 80 pyramidal cells and 20 interneurons was used to simulate oscillatory activity in the model system. Fewer neurons reduced the power and

therefore reliability of gamma oscillations whereas more neurons increased simulation time without increasing gamma power. Differential equations were solved in MATLAB using the midpoint method.

In order to make comparisons with *in vitro* data, equation (4) (Bedard et al., 2010) was used to give an approximation of LFP.

$$V_{LFP} = \frac{R_e}{4\pi} \sum_j \frac{I_j}{r_j} \quad (4)$$

Where R_e is the extracellular resistivity assumed to be $230 \, \Omega \, \text{cm}^{-1}$, I_j is the sum of the currents of cell j , and r_j is taken from a normal distribution representing distance between cell and position where LFP is recorded. A subpopulation of 40 pyramidal cells and 10 interneurons was used to calculate LFP

Table 1. Model parameters

All parameters were based on those used in Kopell et al., 2010. Where changes were made the original values from Kopell et al., 2010 are given in grey brackets for comparison. Changes in sodium and potassium conductance and reversal potentials were chosen to produce waveforms closer to those we observed experimentally and were within the ranges used in other similar biophysical modelling studies (Traub et al., 1994; Wang and Buzsaki, 1996; Ermentrout and Kopell, 1998).

	Parameters		Value		Unit
			Excitatory cells	Inhibitory cells	
Number of cells		N_{pr}	80		
		N_{in}		20	
	Capacitance	C	1	1	$\mu\text{F cm}^{-2}$
Conductances	Sodium	g_{Na}	50 (100)	35	mS cm^{-2}
	Potassium	g_K	20 (80)	9	mS cm^{-2}
	Leak	g_L	0.1	0.1	mS cm^{-2}
	M-current	g_{K_m}	0.5		mS cm^{-2}
Reversal Potentials	Sodium	V_{Na}	50	55	mV
	Potassium	V_K	-70 (-100)	-90	mV
	Leak	V_L	-67	-65	mV
	M-current	V_{K_m}	-85		mV
Synaptic conductances	E to E	g_{E-E}	0.5 (0)		mS cm^{-2}
	I to E	g_{I-E}	1.5		mS cm^{-2}
	I to I	g_{I-I}		0.7 (0.5)	mS cm^{-2}
	E to I	g_{E-I}		1.5 (0.5)	mS cm^{-2}
Synaptic reversal potentials		V_{rev}	0	-80	mV
Synaptic time constants	Rise time	τ_R	0.1	0.3	ms
	Decay time	τ_D	3	9	ms

Analysis

Fast fourier transform (FFT) of LFP data were performed to generate power spectral densities (PSD) using the chronux toolbox in MATLAB and a 5 taper multi-taper estimate. In all experiments baseline PSDs with no stimulation were subtracted from data with stimulation input to remove background noise. In every case baseline and stimulation PSDs were calculated from downsampled data (1 kHz) and taken from an average of six 2.5s frames. Subtracted PSDs were smoothed with a moving average filter with span of 35 data points. Gamma oscillation power was calculated as the integral of the PSD between 30 Hz and 100 Hz and theta oscillation power between 4 Hz and 12 Hz. The peak gamma oscillation frequency was measured at the maximum power between 30 Hz and 100 Hz. Gamma and theta oscillation power was baseline subtracted within each slice experiment.

Statistics

Data are plotted as mean \pm s.e.m. throughout the manuscript. Kolmogorov-Smirnov test showed data were normally distributed. Data for drug concentration comparisons were then analysed by one-way ANOVA followed by post-hoc 2-sample t-tests assuming unequal variances using Holm-Bonferroni correction to test the null hypothesis that data were the same as baseline for each pharmacological manipulation. Experimental numbers for statistical analysis were taken as the number of slices used. * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and no star denotes $p > 0.05$.

Results

Optogenetically-induced gamma oscillations in hippocampal slices.

Excitation of local excitatory and inhibitory networks has been shown to produce gamma frequency oscillations in *in vitro* acute brain slice preparations. In the hippocampus, application of glutamatergic or cholinergic agonists provides excitation that generates gamma oscillations in the 30-50 Hz range (Buhl et al., 1998; Fisahn et al., 1998; Palhalmi et al., 2004). However, these persistent gamma oscillations, although robust and therefore amenable to experimental manipulation, are not observed *in vivo*. More recently, gamma oscillations have been evoked *in vivo* and *in vitro* using transient activation of channelrhodopsin (ChR) to provide excitatory drive to the network. This has been achieved in slices of neocortex or hippocampus by expressing ChR in neurons which are then stimulated by step, ramp or sinusoidal waveforms of light (Adesnik and Scanziani, 2010; Akam et al., 2012; Pastoll et al., 2013; Butler et al., 2016). **In particular, the use of an optogenetically driven theta frequency sine wave produces theta nested high frequency gamma comparable to that found *in vivo*.** The use of optogenetics to induce gamma oscillations provides a system where the modulation of gamma oscillations by pharmacological activation of cholinergic receptors may be tested. Therefore, we developed an optogenetic system for inducing gamma oscillations in the CA3 region of acute hippocampal slices.

ChR expression was targeted preferentially to CA3 PCs by stereotaxic injection of a viral vector (AAV5) containing ChR (hChR2(H134R)) tagged with YFP under the control of the CaMKII α promoter into the dorsal CA3 region at p21 (see methods). YFP expression increased over 35 days post injection (d.p.i, Figure 1A) after which expression levels plateaued and appeared strongest in stratum radiatum due to higher membrane density. Functional ChR expression was assessed by local field potential (LFP) recording in stratum

radiatum in the CA3 area (Figure 1B). 10 ms optical stimulation (470 nm) of a circular area encompassing most of the slice evoked LFP responses comprised of slow (onset >8ms after the start of light stimulation) and fast (onset <5ms after the start of light stimulation) components which were blocked by NBQX (10 μ M) and TTX (1 μ M) respectively and were therefore termed excitatory postsynaptic potentials (EPSPs) and fiber volleys (Figure 1C). A residual LFP response was observed in the presence of TTX due to charge flowing through ChR. EPSP amplitude increased with increasing light intensity which plateaued around 600-800 μ W (Figure 1D).

We then tested the relative efficacy of step, ramp or sinusoidal waveforms of light stimulation for the generation of gamma oscillations in hippocampal slices. A representative example of a comparison made in a single slice is shown in Figure 1E. A 1 s step waveform (Figure 1Ei) elicited gamma oscillations with peak frequency 61.5 Hz but had a low initial power that attenuated rapidly (average power $1.28 \times 10^{-5} \text{ mV}^2$). A ramp stimulus to the same peak light intensity as the step stimulus induced gamma oscillations with similar peak frequency (62.4 Hz) but with higher average power ($2.16 \times 10^{-4} \text{ mV}^2$) and less attenuation (Figure 1Eii). Moreover, gamma oscillation peak frequency did not vary over the course of the ramp stimulation. Hippocampal gamma oscillations *in vivo* are often observed 'nested' within an overlying theta (4-12 Hz) oscillation (Bragin et al., 1995). We mirrored this in our preparation by stimulating the slice with a 5 Hz or 8 Hz sinusoidal waveform that had the same average light intensity as the step waveform (Figure 1Eiii and 1Eiv) (Pastoll et al., 2013). These stimulations reliably elicited gamma oscillations of higher peak frequency (75.7 Hz and 78.7 Hz respectively) and comparable average power ($6.80 \times 10^{-5} \text{ mV}^2$ and $8.90 \times 10^{-5} \text{ mV}^2$) with minimal attenuation. Gamma oscillation peak frequency reduced over sequential theta cycles and therefore mean gamma oscillation peak frequency was calculated across the entire 1 s stimulation period (Average peak frequency for 5 Hz stimulation was 65.7 ± 3.0

Hz, n = 54). In accordance with the YFP expression data (Figure 1A), expression levels of functional ChR were only sufficient to reliably generate gamma oscillations in response to 5 Hz stimulation around 28 d.p.i. and induced gamma oscillation power was consistent for longer periods of expression post injection (average gamma oscillation power $7.98 \times 10^{-5} \pm 1.27 \times 10^{-5} \text{ mV}^2$, range 27 - 53 d.p.i., n = 54). Given the physiological relevance of theta frequency for the generation of gamma oscillations, the 5 Hz sinusoidal waveform stimulation protocol was selected for all further experiments and the maximum light stimulation intensity was set to the value that elicited a half maximal EPSP response (Figure 1D).

In our experimental model, gamma oscillations are hypothesised to be generated by the direct reciprocal interaction between glutamatergic CA3 PCs and GABAergic PV BCs (Adesnik and Scanziani, 2010; Akam et al., 2012; Pastoll et al., 2013; Butler et al., 2016). We tested this by application of glutamatergic or GABAergic antagonists to gamma oscillations induced by 5 Hz, theta frequency light stimulation. The AMPA/kainate receptor antagonist NBQX was bath applied at increasing concentrations from 0.1 to 10 μM (Figure 2A,B). Gamma oscillation power was decreased at concentrations $\geq 1 \mu\text{M}$ without affecting peak frequency and was reversed after washout of NBQX. The GABA_A receptor antagonist picrotoxin (PTX) also decreased the power of gamma oscillations in a dose-dependent and reversible manner without affecting peak frequency (Figure 2C,D). These results support a model where optogenetically-induced theta nested gamma oscillations are generated by reciprocal excitatory and inhibitory synaptic connectivity within the CA3 network (Buzsaki and Wang, 2012).

Modulation of optogenetically-induced gamma oscillations by carbachol.

We next sought to determine whether acetylcholine could modulate gamma oscillations in our experimental system. The non-hydrolysable analogue of acetylcholine, carbachol (CCh), was bath applied at increasing concentrations from 50 nM to 10 μ M. Low concentrations of CCh (0.05 and 0.1 μ M) caused an increase in gamma oscillation power whereas higher concentrations (3 and 10 μ M) caused a decrease in gamma oscillation power (Figure 3A,B). The peak average frequency of gamma oscillations was not affected by any concentration of CCh (Figure 3A,B) and neither was the peak frequency during the first theta cycle nor the attenuation of gamma frequency across consecutive theta cycles (Supplementary Figure 1). The effects of CCh on the power of gamma oscillations was fully reversed on washout of CCh and furthermore, time matched control experiments showed that gamma oscillation power and peak frequency did not vary over the course of experiments (Figure 3C). *In vivo* observations indicate that gamma oscillation power is dependent on theta oscillation power (Bragin et al., 1995). Therefore, we next analyzed whether CCh had any effect on the power of theta oscillations. We found that theta oscillation power remained constant for all concentrations of CCh application (Figure 3D) indicating that CCh-induced changes in gamma oscillation power were not attributable to changes in theta oscillation power. In addition, neither theta nor gamma power were increased outside of optogenetic stimulation by these concentrations of carbachol. These results show that CCh induces a bi-directional dose-dependent effect on the power of gamma oscillations that is independent of underlying theta oscillation power.

Acetylcholine activates nicotinic $\alpha 4\beta 2$, $\alpha 3\beta 4$ and $\alpha 7$ receptors and muscarinic M1, M2, M3 and M4 receptors in the hippocampus causing a range of effects including inhibition of potassium channels and depolarization (M1 and M3) and regulation of presynaptic calcium channels and release of neurotransmitter (M2, M4, $\alpha 4\beta 2$, $\alpha 3\beta 4$ and $\alpha 7$) (Teles-Grilo Ruivo and Mellor, 2013). Acetylcholine and its non-hydrolysable analogue carbachol have different affinities and efficacies at these cholinergic receptor subtypes (Jensen et al., 2003). To investigate the mechanism underlying cholinergic modulation of gamma oscillations and identify which cholinergic receptors are involved we developed a biophysical model of CA3 comprising a network of single compartment Hodgkin-Huxley type neurons, based on the work of Kopell et al., (see methods) (Kopell et al., 2010). 80 pyramidal cells and 20 interneurons were all-to-all connected (Figure 4A), fired action potentials (Figure 4B) and exhibited co-ordinated network behavior (Figure 4C). As previously described in experimental and theoretical studies (Kopell et al., 2010; Akam et al., 2012), step input to the pyramidal cells ($1.25 \mu\text{A cm}^{-2}$) drove the network to fire at gamma frequency (Figure 4Ci) as did theta frequency sinusoidal inputs ($2.5 \mu\text{A cm}^{-2}$ maximum amplitude) at 5 Hz or 8 Hz (Figure 4Cii, Ciii) thus recapitulating key characteristics of the experimental data. To allow comparisons with our experimental results, we used the 5 Hz sinusoidal input in all further simulations which produced gamma oscillations with peak frequency 41.2 Hz and average power $3.94 \times 10^{-4} \text{ mV}^2$.

We next tested two hypothesised effects of acetylcholine on gamma oscillations: (i) depolarisation of PCs caused by activation of a non-selective cationic conductance and inhibition of KCNQ channels (M-current) which model core aspects of muscarinic M1 receptor activation (Madison et al., 1987; Fisahn et al., 2002), and (ii) reduction of inhibitory synaptic transmission which models an aspect of muscarinic M2 and nicotinic $\alpha 4\beta 2$, $\alpha 3\beta 4$

and $\alpha 7$ receptor activation (Alkondon and Albuquerque, 2001; Ji et al., 2001; Szabo et al., 2010; Tang et al., 2011; Teles-Grilo Ruivo and Mellor, 2013). Inhibition of M-current in the model had no effect on gamma oscillation power or frequency (Supplementary Figure 2). To model the effects of activating a non-selective cationic conductance (**which could be voltage or calcium dependent**) a constant depolarising input current of increasing amplitude was applied to PCs in addition to the theta frequency sinusoidal current injection (Figure 5A). 1 $\mu\text{A cm}^{-2}$ constant input current caused PCs to depolarise from -72.3 ± 1.4 mV to -69.9 ± 2.1 mV (Figure 5B) and increased the power of gamma oscillations (Figure 5C, D). As input current was increased, PCs were more depolarised (to -65.3 ± 0.8 mV at 6 $\mu\text{A cm}^{-2}$) (Figure 5B) and the power of gamma oscillations was reduced (Figure 5C,D). The peak gamma oscillation frequency remained relatively unaffected, within a range of input currents, exhibiting only a slight increase (Figure 5D). We then tested the mechanism for the biphasic effect on gamma oscillation power by analysing the firing properties of neurons during theta frequency excitation. 1 $\mu\text{A cm}^{-2}$ input current to PCs caused both PCs and PV BCs to increase their probability of firing across the theta cycle but with the timing of spikes still entrained to gamma frequency (Figure 5E). However, as the input current was increased up to 6 $\mu\text{A cm}^{-2}$ the entrainment to gamma frequency was lost such that PCs, and therefore PV BCs, fired at much higher frequencies and the network became desynchronised (Gulyas et al., 2010) (Figure 5E). These results indicate that activation of M1 mAChRs is sufficient to replicate the experimental data for the biphasic modulation of gamma oscillation power by carbachol and point towards a mechanism involving an increase in non-selective cation conductance rather than inhibition of M-current.

To model the effects of reducing inhibitory synaptic transmission the inhibitory-to-excitatory synaptic conductance (gI-E) was reduced in the model (Figure 6A). Reducing gI-E from 1.5 mS cm^{-2} to 0.9 mS cm^{-2} prevented gamma oscillations below a value of 1.1 mS cm^{-2} (Figure

6B, C). Analysis of neuronal firing during theta frequency stimulation revealed that reducing gI-E below 1.1 mS cm^{-2} caused PV BCs to depolarise sufficiently to inactivate Na^+ channels and therefore stop firing action potentials leading to a complete loss of rhythmic network activity (Figure 6D). These results suggest that a critical level of gI-E is necessary for gamma oscillations but above this threshold gI-E does not modulate either their power or frequency.

Overall, these simulations predict that the principal effect of acetylcholine – namely the bidirectional dose-dependent increase and decrease in gamma oscillation power - could be explained solely by the activation of M1 mAChRs although it is possible that the decrease in gamma oscillation power at higher concentrations could also be partly mediated by activation of other cholinergic receptors that depress inhibitory synaptic transmission.

Interestingly, both the experimental and simulation data showed a remarkable stability in gamma oscillation frequency despite considerable modulation of gamma oscillation power across the range of carbachol concentrations (Figure 3) and current injections (Figure 5) respectively. The frequency of gamma oscillations has been proposed to be principally governed by the activity of, and therefore synaptic input to, interneurons during ongoing gamma oscillations (Whittington et al., 1995; Wang and Buzsaki, 1996; Jadi and Sejnowski, 2014) and therefore we hypothesised that stable gamma oscillation frequency could result from stable synaptic input to interneurons during gamma oscillations. We tested this in the biophysical model by increasing the interneuron-to-interneuron synaptic conductance (gI-I from 0.7 mS cm^{-2} to 1.1 mS cm^{-2}), which produced an increase in the net synaptic current (calculated by summing the excitatory and inhibitory synaptic currents during the simulation period) during gamma oscillations in interneurons but not in pyramidal cells (Figure 7A). Furthermore, net synaptic current remained fairly constant across the range of constant current injections to the pyramidal cells found to modulate gamma oscillation power when $\text{gI-I} = 0.7 \text{ mS cm}^{-2}$ but in contrast, the net synaptic current increased substantially when

gI-I = 1.1 mS cm⁻² as the constant current injection was increased. This suggested that increasing inhibitory synaptic conductance between interneurons reduces the stability of net synaptic current to interneurons during gamma oscillations and therefore the stability of gamma oscillation frequency. Indeed, when we analysed the gamma oscillation frequency stability across the range of depolarising current injections we found that using a low gI-I produced relatively stable frequencies but when gI-I was increased to 1.1 mS cm⁻² or 1.5 mS cm⁻² gamma oscillation frequency was much less stable with increasing depolarising current (Figure 7B and C). Similarly, using an alternative approach to reduce the excitatory-inhibitory synaptic balance to interneurons, we found that reducing excitatory synaptic conductance onto interneurons (gE-I) from 1.5 mS cm⁻² to 1.0 mS cm⁻² or 0.5 mS cm⁻² also produced much less stable gamma oscillation frequencies (Figure 7B and C). These results suggest that the gamma oscillation generating network in CA3 of our acute hippocampal slices contains interneurons with relatively large excitatory compared to inhibitory synaptic inputs.

M1 mAChRs mediate the modulation of gamma oscillations by acetylcholine.

To test the model predictions we used a combination of pharmacological and genetic approaches in the experimental optogenetic model of theta-nested gamma oscillations. The simulations predict that M1 mAChR activation is the principal driver of cholinergic modulation of gamma oscillations so we tested if M1 mAChR activation was sufficient and necessary using a selective M1 mAChR agonist and M1 mAChR knockout mice.

The M1 mAChR allosteric agonist GSK-5 (Budzik et al., 2010; Dennis et al., 2015) was applied to slices in increasing concentrations from 50 nM to 3 μM. In an almost identical manner to CCh (Figure 3), GSK-5 produced a bidirectional dose-dependent change in the

power of gamma oscillations (Figure 8A,B). Gamma oscillation power increased following applications of 50 nM or 100 nM GSK-5 whereas 1 μ M or 3 μ M caused a decrease in power (Figure 8A,B). We have previously found that GSK-5 is not readily removed from slices on washout (Dennis et al., 2015) and this was also true for the effects on gamma oscillation power. Again, similar to CCh, there was no change in peak gamma oscillation frequency at any concentrations of GSK-5 tested (Figure 8B). These results support the mathematical model predictions that M1 mAChRs play a key role in the modulation of gamma oscillations by acetylcholine.

To test whether any other cholinergic receptors are important for the modulation of gamma oscillations we made use of M1 mAChR knock-out (M1KO) mice (Fisahn et al., 2002; Buchanan et al., 2010; Dennis et al., 2015). ChR was expressed in the hippocampus following viral injection using the same methods as wild-type mice and gamma oscillations of similar peak frequency and power were elicited by 5 Hz sinusoidal stimulation. In slices from M1KO mice the effects of CCh application were absent with no increase or decrease in the power or peak frequency of gamma oscillations (Figure 8C,D). These results strongly indicate that M1 mAChRs are the principal mediators of the bidirectional dose-dependent effects of acetylcholine on gamma oscillations.

Discussion

In this study we employed two separate approaches to investigate the modulation of gamma oscillations by acetylcholine. We first developed a model for studying theta-nested gamma oscillations using theta frequency optogenetic stimulation of acute hippocampal slices (Pastoll et al., 2013; Butler et al., 2016). We found that the broad spectrum cholinergic agonist carbachol modulates gamma oscillation power, but not frequency, in a bidirectional

and dose-dependent manner. We then tested the mechanism for this bidirectional modulation using a mathematical biophysical network model for gamma oscillations (Kopell et al., 2010) which predicted that M1 mAChRs were the most likely mediators for the effects of acetylcholine. Finally, we tested the model predictions and found that activation of M1 mAChRs is both sufficient and necessary for the modulation of gamma oscillations by acetylcholine.

Theta-nested gamma oscillations in CA3 region of the hippocampus are generated *in vivo* by the coordinated interactions of excitatory and inhibitory neurons which are triggered by excitation of both groups of neurons phase locked to the theta cycle (Buzsaki and Wang, 2012). This ensures that gamma power strongly covaries with theta power for theta-nested gamma (Bragin et al., 1995). Coordinated excitation of excitatory and inhibitory neurons may also be provided *in vitro* **in a persistent manner by different pharmacological mechanisms including** stimulation of kainate receptors, metabotropic glutamate receptors or muscarinic receptors (Buhl et al., 1998; Fisahn et al., 1998; Palhalmi et al., 2004). Although gamma oscillations are not thought to be triggered directly by activation of these receptors *in vivo*, this strongly suggests that cholinergic receptor activation can modulate theta-nested gamma oscillations. The power of theta oscillations in the hippocampus is modulated by cholinergic innervation (Lee et al., 1994; Vandecasteele et al., 2014) providing one indirect mechanism for the modulation of gamma oscillations by acetylcholine. In contrast, we demonstrate a direct mechanism for the modulation of gamma oscillations where theta oscillation power and frequency remain constant.

We found that carbachol modulates the power of gamma oscillations in a bidirectional manner which initially suggested two distinct modulatory mechanisms mediated by multiple cholinergic receptors with different efficacies. Two potential mechanisms are an increase in excitability caused by activation of M1/M3 mAChRs and the decrease in inhibitory synaptic

conductance caused by activation of M2 mAChRs or $\alpha 4\beta 2$, $\alpha 3\beta 4$ or $\alpha 7$ nicotinic receptors.

Indeed, the power and frequency of persistent gamma oscillations generated by kainate have been shown to be modulated by nicotinic receptors (Wang et al., 2015). However, the frequency of gamma oscillations was unchanged by carbachol. This was surprising because acetylcholine has effects on both PV BC excitability and inhibitory synaptic transmission (Alkondon and Albuquerque, 2001; Ji et al., 2001; Szabo et al., 2010; Tang et al., 2011; Teles-Grilo Ruivo and Mellor, 2013; Yi et al., 2014) which are key determinants of gamma oscillation frequency (Whittington et al., 1995; Wang and Buzsaki, 1996; Mann and Mody, 2010; Oren et al., 2010; Jadi and Sejnowski, 2014). Therefore, our data indicate a limited effect of carbachol on gamma oscillations via modulation of inhibition possibly due to a lower potency or efficacy at the receptor subtypes regulating inhibition (Jensen et al., 2003).

In addition, our modelling predicts that networks with high excitatory to inhibitory synaptic input ratio will be resistant to modulations in gamma oscillation frequency (Figure 7) whereas those with a lower ratio will be less resistant suggesting that different networks may vary in their frequency modulation. The lack of a role for M3 mAChRs, which might otherwise be expected to perform a similar role to M1 mAChRs, is supported by the differential functional expression and distribution of M3 and M1 mAChRs within the hippocampus (Porter et al., 2002; Dennis et al., 2015). Indeed, we found that the bidirectional modulation of gamma oscillations could be explained entirely by enhancing the excitability of PCs caused by activation of M1 mAChRs.

Optogenetic theta frequency stimulation of entorhinal cortex or hippocampus generates theta-nested gamma oscillations that exhibit many important properties found in naturally occurring theta-nested gamma including the relative timing of excitatory and inhibitory neurons within the network (Pastoll et al., 2013; Butler et al., 2016). In addition, since theta oscillation frequency and power may be kept constant, optogenetically stimulated theta-

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nested gamma oscillations make an excellent model system to assess the modulation of gamma oscillations. Similarly, the mathematical model enabled us to probe the mechanism for cholinergic modulation of gamma oscillations by varying the known biophysical properties of cholinergic receptor subtype activation producing predictions which were subsequently tested experimentally revealing the key role for M1 mAChRs. Although the mathematical model was based on experimental biophysical parameters and recapitulated many of the core experimental observations it did diverge in some respects to the experimental model most notably in the average gamma oscillation frequency which was lower in the mathematical model **and may indicate potential differences between gamma oscillation mechanisms exhibited in the mathematical and experimental models.** However, the predictions provided by the mathematical model were confirmed in the experimental model indicating the validity of the mathematical modelling approach.

Entrainment of neuronal activity to gamma oscillations is thought to be critical for local circuit computations and the transfer of information between brain regions (Colgin et al., 2009; Sohal et al., 2009; Ainsworth et al., 2012). The coherence and frequency of gamma oscillations are therefore critical for cognitive processing. Neuromodulators represent an excellent mechanism for modulating gamma oscillations and therefore regulating cognitive processing at a local and global level. However, the mechanisms by which neuromodulators modulate gamma oscillations are poorly understood. In this study we reveal that the neuromodulator acetylcholine modulates the power but not frequency of gamma oscillations in the hippocampus. This mechanism could work in tandem with the modulation of theta oscillations to mediate the effects of acetylcholine on cognition (Lee et al., 1994; McGaughy et al., 2000; Vandecasteele et al., 2014; Okada et al., 2015). Indeed optogenetically induced acetylcholine release enhances theta and gamma power in the hippocampus of anaesthetized mice but reduces power in both bands in awake mice (Vandecasteele et al., 2014).

Acetylcholine is released tonically in the hippocampus at high levels during performance on cognitively demanding tasks and to a lesser extent during REM sleep but is generally very low during non-REM sleep (or in the anaesthetized state) (Marrosu et al., 1995; Teles-Grilo Ruivo et al., 2017). These observations suggest that gamma oscillations may be regulated in a bidirectional manner dependent on behavioural state.

Disruption to gamma oscillations is a prominent feature of schizophrenia and Alzheimer's disease. Cognitive control processes correlate with modulation of gamma oscillations in healthy humans but this modulation is absent in schizophrenia (Cho et al., 2006). The disruption to gamma oscillations in schizophrenia is believed to result from a reduction in PV BC numbers and connectivity (Gonzalez-Burgos et al., 2015). In parallel, disruptions to gamma oscillations in Alzheimer's disease are also thought to follow from a deficit in PV BCs and reversing these PV BC deficits in animal models reduces the associated cognitive deficits (Verret et al., 2012). An alternative strategy for cognitive enhancement could involve modulation of gamma oscillations by agents that target neuromodulator systems. In this study we identify one such target as the M1 mAChR. Interestingly, M1 agonists can enhance cognition in animals and human models of cognitive deficit (Bodick et al., 1997; Shirey et al., 2009; Digby et al., 2012; Nathan et al., 2013) supporting M1 mAChRs as a potential therapeutic target. Furthermore, our data suggest that the effects of any M1 mAChR agonist will be strongly dose-dependent. This, and the high M1 mAChR reserve in the hippocampus (Porter et al., 2002), will be important factors for the future development of M1 agonists as cognitive enhancers.

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Author Contributions:

RTB, KT-A and JRM designed the study; RTB performed experiments and modelling and analysed the data; LMB provided reagents; KT-A and JRM supervised the project; RTB, LMB, KT-A and JRM wrote the paper.

Data Accessibility:

All primary data are archived at the University of Bristol and available on request.

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Figure Legends

Figure 1. Optogenetic induction of gamma oscillations.

A. Expression of ChR2-EYFP over time (d.p.i.in top left of each image) after injection of rAAV5-CaMKIIa-hChR2 (H134R)-EYFP virus into CA3 region Scalebar is 500 μ m. B. Schematic diagram illustrating recording electrode placement within stratum radiatum in CA3 of acute hippocampal slice. C. Example LFP responses to 10ms light stimulation (470nm, 581 μ W) during control and following NBQX (10 μ M) or TTX (1 μ M) application. D. Amplitude of the LFP response plotted against light intensity (n = 10 slices from 8 animals). E. Comparison of optogenetic protocols for induction of gamma oscillations. First row: Schematic of light stimulation protocol. Column i: 1 s step; column ii: 1 s ramp; column iii: 5 Hz sine wave; column iv: 8 Hz sine wave. Subsequent rows show unfiltered LFP, LFP bandpass filtered between 30 - 100 Hz, power spectral density plots and spectrograms (maximum power given in top right). Data shown are from a single slice.

Figure 2. Inhibition of excitatory and inhibitory synaptic connections reduces the power of theta-nested gamma oscillations.

A. Example unfiltered (top) and bandpass filtered (bottom) LFP traces showing response to 5 Hz sinusoidal light stimulation before, during and after NBQX (10 μ M). B. Gamma oscillation power but not peak frequency was reduced by NBQX at concentrations of 3 and 10 μ M. (n = 9 slices from 6 animals; p = 5.89×10^{-5} and 1.92×10^{-8} compared to baseline for 3 and 10 μ M respectively, p > 0.05 for all other concentrations). C. Example unfiltered (top) and bandpass filtered (bottom) LFP traces showing response to 5 Hz sinusoidal light stimulation before, during and after picrotoxin (PTX, 10 μ M). D. Gamma oscillation power but not peak frequency was reduced by picrotoxin at concentrations of 3, 10 and 30 μ M. (n = 8 slices from 6 animals; p = 0.00265, 0.00204 and 0.00349 compared to baseline for 3, 10 and 30 μ M respectively, p > 0.05 for all other concentrations). ** p<0.01, *** p<0.001.

Figure 3. Carbachol induces a dose-dependent bidirectional change in the power of theta-nested gamma oscillations.

A. Example unfiltered (top) and bandpass filtered (2nd row) LFP traces showing response to 5 Hz sinusoidal light stimulation before, during and after carbachol (0.1 and 10 μ M). Power spectral density plots and spectrograms (maximum power given in top right) are shown below for an example experiment. B. Gamma oscillation power but not peak frequency was increased at low concentrations (0.1 μ M) of carbachol but reduced at higher concentrations (3 and 10 μ M) (n = 11 slices from 9 animals; p = 0.00383, 0.00350 and 5.950×10^{-8} compared to baseline for 0.1, 3 and 10 μ M respectively, p > 0.05 for all other concentrations). C. Gamma oscillation power and peak frequency were unchanged in time matched control experiments (n = 4 slices from 3 animals; p > 0.05 for all time points). D. Theta oscillation

power was not changed by any concentration of carbachol ($n = 11$ slices from 9 animals; $p > 0.05$ for all concentrations). ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Theta-nested gamma oscillations in a biophysical network model.

A. Schematic representation for model connectivity between excitatory (E_N) and inhibitory (I_N) neurons. B. Spiking characteristics of excitatory (top, red) and inhibitory (bottom, blue) neurons within the network. C. Network behaviour in response to step (Ci) or 5 Hz (Cii) or 8 Hz (Ciii) input to excitatory cells with maximal amplitude $2.5 \mu A \text{ cm}^{-2}$. 1st row: Schematic of current injection protocol; 2nd row: raster plot of spiking for a network of 80 excitatory (red) and 20 inhibitory (blue) neurons; 3rd row: LFP; 4th row: power spectral density plots; 5th row: spectrograms (maximum power given in top right).

Figure 5. Modelling the effect of M1 mAChR activation on gamma oscillations.

A. Schematic representation of depolarizing current given to excitatory neurons within the network to model the action of M1 mAChRs. B. Increasing the amount of current injection depolarised pyramidal neurons in the mathematical model. C. Power spectral density plots for increasing current injections. D. Gamma oscillation power was increased at low current injection ($1 \mu A \text{ cm}^{-2}$) but reduced at higher current injections (4 and $6 \mu A \text{ cm}^{-2}$) ($n = 7$; $p = 2.24 \times 10^{-4}$, 5.09×10^{-4} , 7.82×10^{-10} and 5.56×10^{-11} compared to baseline for 1, 2, 4, 6 and $8 \mu A \text{ cm}^{-2}$ respectively). E. Example spiking output during sinusoidal input to pyramidal cells (black trace overlaid) for excitatory (top) and inhibitory (bottom) neurons over the range of constant current injections given to pyramidal neurons. *** $p < 0.001$.

Figure 6. Modelling the effect of M2 mAChR activation on gamma oscillations.

A. Schematic representation of reduction in inhibitory-excitatory synaptic conductance (gI-E) within the network to model the action of M2 mAChRs. B. Power spectral density plots for reduced gI-E. C. Gamma oscillation power was decreased for reduced gI-E (1.0, and 0.9 mS cm⁻²) (n = 7; p = 1.59 x 10⁻²⁵ and 1.37 x 10⁻²³ compared to baseline for 1.0 and 0.9 mS cm⁻² respectively). D. Example spiking output during sinusoidal input to pyramidal cells (black trace overlaid) for excitatory (top) and inhibitory (bottom) neurons over the range of gI-E. *** p<0.001.

Figure 7. Gamma oscillation frequency stability is governed by synaptic input to interneurons.

A. Net synaptic current density (excitatory synapses are characterised by positive current and vice versa) during modelled gamma oscillations induced by theta frequency stimulation became more strongly negative in pyramidal cells (left) but less so in interneurons (right) as the constant depolarising current increased. Increasing inhibitory-to-inhibitory conductance, gI-I, from 0.7 mS cm⁻² to 1.1 mS cm⁻² produced no change in net synaptic current density in pyramidal cells but became more negative in interneurons particularly with large constant depolarising current. B. Gamma oscillation frequency was less stable across a range of constant current injections when excitatory-inhibitory synaptic balance was reduced. In control conditions (gI-I = 0.7 mS cm⁻², gE-I = 1.5 mS cm⁻²) gamma oscillation frequency increased only slightly with applied depolarising current (fitted plots (left) and slopes (right)). When E-I balance was altered by either increasing gI-I or reducing gE-I gamma oscillation frequency increased strongly with applied depolarising current (n = 7; p = 8.71 x10⁻³, 6.77 x10⁻⁴, 5.10 x10⁻³, 2.54 x10⁻⁸ for slope comparison control to gI-I = 1.1 mS cm⁻², gI-I = 1.5 mS cm⁻², gE-I = 1.0 mS cm⁻², gE-I = 0.5 mS cm⁻² respectively).

Figure 8. M1 mAChRs are necessary and sufficient for the effects of carbachol on theta-nested gamma oscillations.

A. Power spectral density plots for theta-nested gamma oscillations with increasing concentrations of the M1 mAChR selective agonist GSK-5. B. Gamma oscillation power but not peak frequency was increased at low concentrations of GSK-5 (0.05 μ M) but reduced at higher concentrations (1 and 3 μ M) ($n = 9$ slices from 6 animals; $p = 0.0164$, 5.90×10^{-3} and 7.83×10^{-4} compared to baseline for 0.05, 1 and 3 μ M respectively, $p > 0.05$ for 0.1 and 0.3 μ M; $p > 0.05$ for peak frequency at all concentrations). C. Power spectral density plots for theta-nested gamma oscillations with increasing concentrations of carbachol in slices from M1 KO mice. D. There was no effect of carbachol on gamma oscillation power or peak frequency at any concentration of carbachol ($n = 9$ slices from 6 animals; $p > 0.05$ for gamma power and peak frequency at all concentrations). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.















